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Postconditioning protects rabbit hearts through a protein kinase C-adenosine A_{2b} receptor cascade

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Abstract

Objective: Ischemic postconditioning protects the reperfused heart from infarction, and this protection is dependent on the occupancy of adenosine receptors. We further explored the role of adenosine receptors in this salvage.
Methods: In situ rabbit hearts underwent 30 min of regional ischemia and 3 h of reperfusion, and postconditioning was effected with four cycles of 30-s reperfusion/30-s coronary artery occlusion at the end of ischemia.
Results: Postconditioning reduced infarct size from 40.2 ± 3.4% of the risk zone in untreated hearts to 15.5 ± 2.5%. Protection by postconditioning was blocked by either the non-selective adenosine receptor blocker 8-*p*-(sulfophenyl)theophylline or the A_{2b}-selective antagonist MRS 1754, injected intravenously 5 min before reperfusion. The protein kinase C (PKC) antagonist chelerythrine also aborted postconditioning's salvage, indicating a PKC-dependent mechanism. Neither the A_1-selective antagonist 8-cyclopentyl-1,3-dipropylxanthine nor the A_{2a}-selective antagonist 8-(13-chlorostyryl)caffeine had an effect on protection. The non-selective but A_{2b}-potent adenosine agonist 5′-(*N*-ethylcarboxamido)adenosine (NECA) infused from 5 min before to 1 h after reperfusion mimicked postconditioning's effect on infarct size (17.2 ± 2.7% infarction) and MRS 1754 blocked the NECA-induced cardioprotection, confirming that A_{2b} activation was protective. The PKC activator phorbol 12-myristate 13-acetate delivered just before reperfusion also duplicated the protective effect of postconditioning (16.3 ± 4.1% infarction), and co-administration of the PKC antagonist chelerythrine aborted PMA's protection, confirming that the protection was the result of PKC activation. NECA's protective effect was not affected by chelerythrine, but rather MRS 1754 blocked PMA's salutary effect (42.8 ± 1.0% infarction), suggesting that the A_{2b} receptor's effect is under control of PKC. Finally, wortmannin, a blocker of phosphatidylinositol 3-kinase, also abrogated protection by PMA.
Conclusions: Salvage of ischemic myocardium by postconditioning is dependent on activation of A_{2b} receptors, which in turn depends on activation of PKC. It is still unclear why PKC activation is required to make the heart's adenosine become protective.

Keywords: Adenosine receptors; Ischemia/reperfusion; NECA; PKC; Postconditioning

1. Introduction

Recently, it has been shown that the canine heart can be effectively protected from infarction with brief cycles of ischemia and reperfusion during the early reperfusion period following a lethal ischemic insult, a phenomenon termed postconditioning [1]. We were also able to demonstrate sparing of infarction by postconditioning in hearts of open-

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chest rabbits and showed that this protection was dependent on signal transduction elements leading to nitric oxide (NO) production, ERK activation and opening of the mitochondrial K_{ATP} channel [2]. Tsang et al. [3] extended those observations in a rat model and showed that postconditioning protects myocardium by activating the prosurvival kinases phosphatidylinositol 3-kinase (PI3-K), Akt, and p70S6K.

It is not known what triggers these pathways in the postconditioned heart. One obvious candidate is adenosine which we have previously shown activates PI3-K to increase Akt phosphorylation in the rabbit heart [4]. Furthermore, like postconditioning, protection from the adenosine agonist NECA has been shown to also be dependent on PI3-K, ERK and NO [5]. Indeed, we [6] and Kin et al. [7] reported that postconditioning's cardioprotection in an isolated rabbit heart model can be blocked by the non-selective adenosine receptor antagonist 8-*p*-(sulfophenyl)theophylline (SPT). Ischemic preconditioning was also found to be dependent on adenosine receptor stimulation in the reperfusion phase following the index ischemia [8]. Moreover in that study we presented evidence that the adenosine A_{2b} receptor subtype was largely responsible for preconditioning's protection and that the A_{2a} subtype was not. Although Kin et al. [7] concluded that A_{2a} receptors were responsible for postconditioning's protection, ZM241385, the antagonist they used, is not highly selective between A_{2a} and A_{2b} receptors and it is possible that their conclusion may have been erroneous. We therefore designed experiments to determine whether the A_{2b} receptor subtype might be responsible for postconditioning's protection. In addition, we also explored the mechanism whereby postconditioning causes adenosine receptors to become protective.

2. Methods

All procedures were approved by the Institutional Animal Care and Use Committee and were conducted in accordance with recommendations published in the *Guide for Care and Use of Laboratory Animals* [9].

2.1. Surgical procedure

New Zealand White rabbits of either sex weighing between 1.7 and 2.7 kg were anesthetized with intravenous sodium pentobarbital (30 mg/kg). Throughout the experiment additional anesthesia was administered as needed (5–15 mg pentobarbital/15 min). A heating pad maintained rectal temperature between 38.5 and 39.5 °C. Animals were intubated through a tracheotomy and ventilated with 100% O_2 with the aid of a mechanical ventilator. Arterial pH, pO_2, and pCO_2 were measured with blood gas analyzer ABL 5 (Radiometer, Copenhagen, Denmark). Respiratory rate was adjusted to keep pH between 7.35 and 7.45. In this preparation arterial pO_2 averages 180–200 mm Hg and pCO_2 25–30 mm Hg. A PE 50 catheter filled with heparinized saline (10 U/ml) was placed in the right carotid artery to monitor arterial blood pressure and to measure arterial pH and gases. To administer drugs, a PE 50 catheter filled with heparinized saline was placed in an ear vein.

After a left thoracotomy, a prominent branch of the left coronary artery was surrounded with a suture (2–0 silk) to form a snare. The rabbits were allowed to stabilize for 15 min after surgery before the protocols were begun.

2.2. Protocols

Hearts of 21 experimental groups experienced 30 min of regional ischemia (Fig. 1). In all hearts reperfusion following the occlusion lasted for 3 h. Control hearts had only the index occlusion followed by reperfusion. In all postconditioned groups, four cycles of 30-s reperfusion/30-s occlusion were started immediately after release of the index ischemia. In drug-treated animals SPT (7.5 mg/kg), MRS 1754 (9.5 μg/kg), an adenosine A_{2b}-selective antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 1 mg/kg), a selective A_1 adenosine receptor antagonist, chelerythrine (5 mg/kg), a highly selective antagonist of protein kinase C (PKC), or wortmannin (60 μg/kg), an inhibitor of PI3-K, was infused as an intravenous bolus 5 min before the onset of reperfusion with or without subsequent postconditioning cycles. A 6-min infusion of 8-(13-chlorostyryl)caffeine

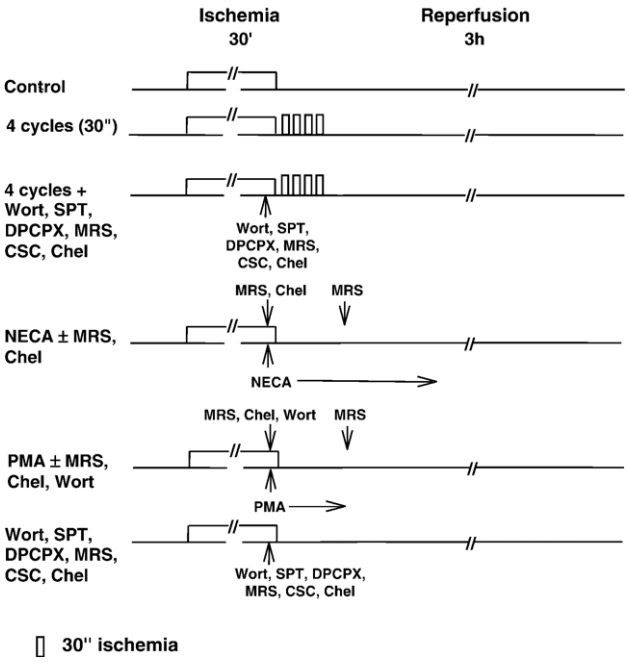

Fig. 1. Experimental protocols. Abbreviations: Chel = chelerythrine; CSC = 8-(13-chlorostyryl)caffeine; DPCPX = 8-cyclopentyl-1,3-dipropylxanthine; MRS = MRS 1754; NECA = 5′-(*N*-ethylcarboxamido)adenosine; PMA = phorbol 12-myristate 13-acetate; SPT = 8-*p*-(sulfophenyl)theophylline; Wort = wortmannin.

(CSC, 660 μg/kg), a selective A_{2a} adenosine receptor antagonist, was started 11 min before the onset of reperfusion and was also combined with postconditioning.

NECA (2 μg/kg), a non-selective but potent A_{2b} adenosine agonist, was infused 5 min before the onset of reperfusion. After administration of the bolus, an infusion of 0.2 μg/kg/min was continued for 65 min. To investigate if the adenosine A_{2b} receptor were responsible for the effects of NECA, we administered a bolus of MRS 1754 (9.5 μg/kg) at the same time as NECA 5 min before the onset of reperfusion and again after 15 min of reperfusion. To determine whether PKC activation was critical to NECA's effect on ischemic myocardium, chelerythrine (5 mg/kg) was infused as an intravenous bolus 5 min before the onset of reperfusion.

The effect of a left atrial infusion of the PKC activator phorbol 12-myristate 13-acetate (PMA) (0.5 μg/kg/min) beginning 5 min before reperfusion and extending for 20 min in lieu of NECA was examined. Co-infusion of either chelerythrine or MRS 1754 as detailed above was also tested in 2 other groups of rabbit hearts.

Finally, the effects of the tool drugs themselves, SPT, MRS 1754, DPCPX, CSC, chelerythrine, and wortmannin infused 5 min before reperfusion were evaluated.

Doses of drugs were selected mainly because of our prior experience of effective action or blockade. DPCPX at a dose of 1 mg/kg had previously been shown to block the negative chronotropic effect of the selective adenosine A_1 receptor agonists 2-chloro-N^6-cyclopentyladenosine [10] and GR79236 [11]. Preliminary observations with CSC demonstrated that a dose of 660 μg/kg infused over 6 min completely prevented the hypotensive effect of an intravenous infusion of 0.4 mg/kg/min adenosine.

2.3. Determination of area at risk and infarct size

After completion of the studies all in situ hearts were excised and the aortic root was perfused with 0.9% saline. The coronary artery was reoccluded, and 2–9 μm diameter green fluorescent microspheres (Duke Scientific Corp., Palo Alto, CA) were infused into the perfusate to demarcate the ischemic zone as the area of tissue without fluorescence (region at risk). Hearts were weighed, frozen, and then cut into 2-mm-thick transverse slices. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) in sodium phosphate buffer (pH 7.4) at 38 °C for 8 min. TTC stains the noninfarcted myocardium brick red indicating the presence of dehydrogenase enzymes. The slices were then immersed in 10% formalin to enhance the contrast between stained (viable) and unstained (necrotic) tissue. The risk zone was revealed as the non-fluorescent region by illumination with UV light. The areas of infarct and risk zone were determined by planimetry of each slice and volumes were calculated by multiplying each area by its slice thickness and summing them for each heart. Infarct size is expressed as a percentage of the risk zone.

2.4. Chemicals

Wortmannin, chelerythrine, PMA, SPT, NECA, MRS 1754, CSC, and DPCPX were obtained from Sigma Chemical Co. (St. Louis, MO). They were dissolved in DMSO. The solution was then further diluted in 1.5 ml of 0.9% saline before administration to the rabbit. This amount of DMSO had no effect in control hearts.

2.5. Statistics

All data are expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) combined with Tukey's post hoc test was performed on baseline hemodynamic and infarct data. Temporal differences in hemodynamic variables in any given group were analyzed with one-way repeated measures ANOVA, and Tukey's post hoc test was used to examine differences between measurements at any given time point and baseline. Differences were considered to be significant if the p value was below 0.05.

3. Results

3.1. Hemodynamics

No group differences in heart rate, developed pressure, or coronary flow were observed at baseline (Table 1 in the online supplement). No substantial changes in heart rate or blood pressure were observed after administration of drugs. Blood pressure declined mildly during coronary occlusion in all groups.

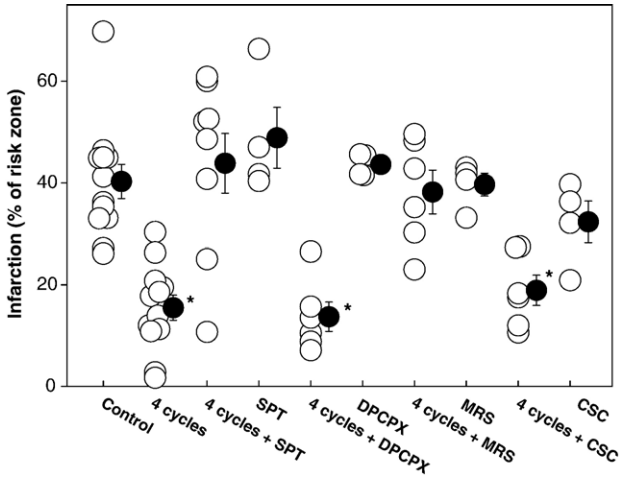

Fig. 2. Infarct size in in situ rabbit hearts. All animals underwent a 30-min coronary artery occlusion and 3-h reperfusion. Open circles represent individual experiments and closed circles depict group means with S.E.M. Four postconditioning cycles protected against ischemia/reperfusion. Both 8-p-(sulfophenyl)theophylline (SPT) and MRS 1754 (MRS), a selective A_{2b} blocker, completely aborted the protection of postconditioning, whereas DPCPX (8-cyclopentyl-1,3-dipropylxanthine) and CSC [8-(13-chlorostyryl)caffeine] had no effect. *$p < 0.001$ vs. control.

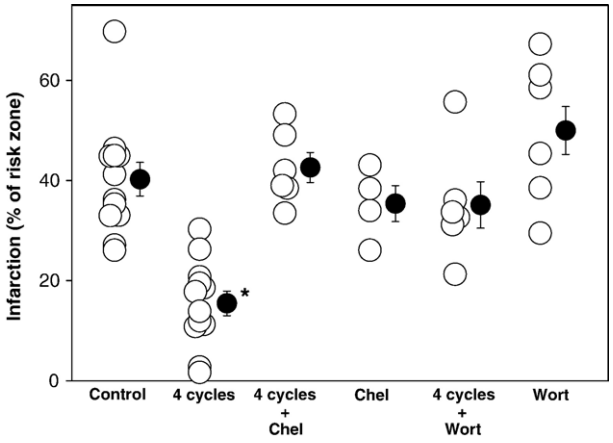

Fig. 3. Infarct size in in situ rabbit hearts. All animals underwent a 30-min coronary artery occlusion and 3-h reperfusion. Open circles represent individual experiments and closed circles depict group means with S.E.M. The protection of postconditioning was blocked by both chelerythrine (Chel) and wortmannin (Wort) infused 5 min before reperfusion. *$p<0.001$ vs. control.

3.2. Infarct size

Although there was some variability in heart weights and risk zone volumes in the groups (Table 2 in the online supplement), there were no significant differences. In control hearts infarct size was 40.2±3.4% of the risk zone (Fig. 2). Infarct size in the postconditioned group was nearly 70% less than that in the control group (15.5±2.5%, $p<0.001$ versus control). SPT, a non-selective adenosine receptor blocker, aborted protection confirming the involvement of adenosine receptors. Furthermore MRS 1754, a highly selective adenosine A_{2b} receptor antagonist, also blocked postconditioning's protection, thus identifying the A_{2b} receptor subtype as being responsible for postconditioning's protection. Neither DPCPX nor CSC, highly selective A_1 and A_{2a} antagonists, respectively, blocked postconditioning's protection, thus making involvement of the A_1- and A_{2a}-receptors unlikely.

We next examined whether PKC activation played a role in postconditioning's protection. As demonstrated in Fig. 3, chelerythrine blocked salvage of ischemic myocardium (42.6±3.0% infarction). Additionally wortmannin, an antagonist of PI3-K, also aborted postconditioning's protective effect (35.1±4.6% infarction).

It was reasoned that if postconditioning protects by activating A_{2b} receptors then administration of an A_{2b} agonist should mimic the protection. Fig. 4 shows that an infusion of the non-selective but A_{2b}-potent adenosine receptor agonist NECA beginning just before reperfusion significantly decreased infarction to 17.2±2.7% of the risk zone ($p<0.001$ vs. control). As predicted by our hypothesis this protection was aborted when A_{2b} receptors were blocked by MRS 1754, thus documenting the importance of A_{2b} receptor occupancy which is presumably the first step in NECA's signaling. Chelerythrine had no effect when protection was initiated by NECA, thus excluding PKC as a necessary step in NECA's downstream signaling pathway.

Next we tried to activate cardiac PKC by directly infusing PMA into the rabbit just prior to reperfusion and found that it was equally protective as NECA (16.3±4.1% infarction) (Fig. 5). Chelerythrine blocked PMA's salutary effect, thus supporting our assumption that PMA protected by activating PKC. The A_{2b} blocker MRS 1754 also blocked PMA's protection indicating that PKC activation acts to control adenosine's activation of the A_{2b} receptor in the signaling cascade. Finally, wortmannin also abrogated PMA's protection.

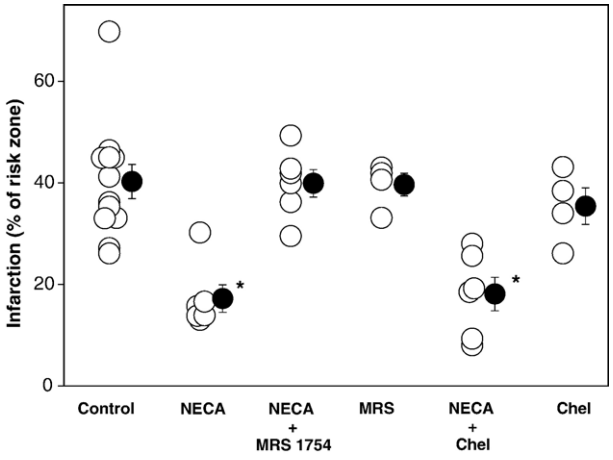

Fig. 4. Infarct size in in situ rabbit hearts. All animals underwent a 30-min coronary artery occlusion and 3-h reperfusion. Open circles represent individual experiments and closed circles depict group means with S.E.M. 5′(*N*-Ethylcarboxamido)adenosine (NECA) administered 5 min before reperfusion protected ischemic hearts, and the adenosine A_{2b} receptor antagonist MRS 1754 (MRS) completely blocked that protection. On the other hand, chelerythrine (Chel) administered just before reperfusion failed to affect NECA's protection. *$p<0.001$ vs. control.

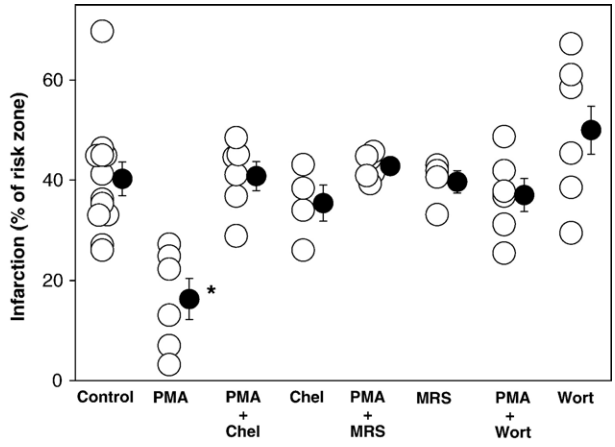

Fig. 5. Infarct size in in situ rabbit hearts. All animals underwent a 30-min coronary artery occlusion and 3-h reperfusion. Open circles represent individual experiments and closed circles depict group means with S.E.M. Phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, administered just before reperfusion salvaged ischemic myocardium. Chelerythrine (Chel), a PKC antagonist, MRS 1754 (MRS), an adenosine A_{2b} antagonist, and wortmannin (Wort), an inhibitor of PI3-K, aborted protection. *$p<0.001$ vs. control.

None of the tool drugs administered at reperfusion had any independent effect on infarct size (Figs. 2 and 3).

4. Discussion

These studies implicate involvement of the adenosine A_{2b} receptor in postconditioning's signaling pathways in our rabbit model. Furthermore for the first time we provide evidence that PKC activation is upstream of the critical binding of adenosine A_{2b} receptors. These observations provide a link back to PKC which for many years has been a pivotal kinase in the protection from ischemic preconditioning [12,13].

Although ischemic preconditioning introduced almost 20 years ago is a powerful anti-infarct intervention [14], it has not been possible to apply it in patients with acute myocardial infarction because of the requirement for pretreatment. Furthermore, restoration of blood flow to ischemic myocardium, a requisite for long-term survival of tissue, appears to hasten the transition from ischemic but viable to necrotic myocardium [15–18]. Zhao et al. [1] provided a new tool for protection of the heart from ischemia/reperfusion injury when they introduced the concept of postconditioning. Because of its timing postconditioning could be applied in a clinical setting of reperfusion by direct angioplasty, and indeed a recent clinical trial of postconditioning yielded encouraging results [19].

Because a highly selective A_{2b} antagonist blocked postconditioning's protection we tested whether an A_{2b} agonist would mimic the protection. Unfortunately a selective A_{2b} agonist is unavailable. Although not selective for any adenosine receptor, NECA is a very potent A_{2b} receptor agonist, and is frequently employed to activate this receptor subtype. As previously demonstrated in isolated rabbit hearts [5], NECA was quite protective in in situ hearts. Therefore, it was not surprising that blockade of A_{2b} receptors with MRS 1754 aborted NECA's protection. Because PKC is known to play an important mediator role in preconditioning, and because a PKC antagonist blocked postconditioning's protection in the present study, we tested whether this intracellular kinase would also be important in NECA's signaling. To our surprise it was not, suggesting that PKC resides upstream of the step where the postconditioned heart employs A_{2b} receptors in its protective pathway.

We pursued the above hypothesis by testing whether activating PKC with PMA would be protective. Intraatrial PMA just prior to reperfusion was equally protective as NECA. We excluded the possibility that PMA was protecting ischemic myocardium through some unknown, nonspecific effect by documenting abolition of PMA's salvage with the highly selective PKC antagonist chelerythrine. The observation that blockade of A_{2b} receptors with MRS 1754 also prevented PMA from sparing ischemic myocardium corroborated our hypothesis that PKC resides upstream of the A_{2b} step in this pathway. Therefore, we propose that PKC activation by postconditioning somehow facilitates the activation of protective signal transduction pathways that are coupled to the A_{2b} receptor.

Kin et al. [7] recently reported that adenosine receptors were involved in a rat model of postconditioning, but they concluded that it was the A_{2a} and A_3 receptor subtypes that were important. AMP579, an adenosine A_1/A_2 agonist, is quite protective when delivered just prior to or at reperfusion, and its cardioprotection was not affected by the potent and selective A_1 adenosine receptor antagonist DPCPX (K_i=10 nM for A_1 and 1440 nM for A_2 receptors), but was effectively abrogated by the adenosine A_{2a} antagonist 8-(13-chlorostyryl) caffeine (CSC) which is 520-fold more selective for A_{2a} than A_1 receptors [20,21]. Yet we were unable to duplicate its protection with the highly selective A_{2a} agonist CGS21680 [21]. These observations led us to suspect the A_{2b} receptor might be the important one since NECA, a very similar compound with known adenosine A_{2b} agonist potency, mimicked AMP579's protection [21] (no A_{2b} affinity data are available for AMP579). Kin et al. [7] used ZM241385 to block A_{2a} receptors in their rat hearts. However, if the affinity of rat receptors for ZM241385 is similar to that of cloned human adenosine receptors [22], then nearly all A_{2b} receptors would also have been blocked as well at the dose used by Kin et al. We were unable to find affinity data for ZM241385 against rat A_{2b} receptors.

Affinity studies for MRS 1754 have been performed for human and rat, but unfortunately not for rabbit, adenosine receptors [23]. The K_i for hA_{2b} is 1.97 nM which is more than 200-fold lower than that for either hA_1 or hA_{2a} receptors. However, the K_i for rat A_1 receptors is 16.8 nM, only 8.5 times that for hA_{2b} receptors. Because of the possible collateral effect of MRS 1754 on A_1 receptors in the rabbit, we specifically examined the result of DPCPX administration at a dose known to block the heart rate effects of a highly selective adenosine A_1 agonist in rabbits [10,11]. Indeed, the A_{2b} antagonist MRS 1754, but not the A_1 antagonist DPCPX, aborted postconditioning's protection, strongly suggesting that cardioprotection from postconditioning in the open-chest rabbit requires occupancy of A_{2b} receptors during reperfusion. Our observations with CSC exclude involvement of the A_{2a} receptor. Unfortunately, there is no selective A_3 antagonist in the rabbit. Interestingly, Kilpatrick et al. [24] have shown that DPCPX blocks cardioprotection triggered by A_3 receptor stimulation in rabbit hearts. Because of the inability of DPCPX to block postconditioning's protection, a role for A_3 receptors is doubtful.

Olanrewaju and Mustafa [25] have demonstrated in coronary artery endothelial cells that occupancy of both adenosine A_{2a} and A_{2b} receptors stimulates NO production and increases cGMP levels. We have already shown that cardioprotection triggered by NECA at reperfusion in vitro [5] as well as postconditioning [2] are dependent on NO.

Mitochondrial K_{ATP} opening has also been implicated in postconditioning [2]. Increased PKG activation resulting from an increased cGMP level would be expected, and we have recently reported that activated PKG can open mitochondrial K_{ATP} [26].

Tsang et al. [3] reported that PI3-K is an important signaling step in postconditioning in the isolated rat heart. We now confirm this observation in the in situ heart, and also show that PMA's protection is effectively blocked by wortmannin. Previously, we had noted that salvage triggered by NECA infused shortly before reperfusion was blocked by wortmannin [5]. Furthermore, NECA increased Akt phosphorylation. Therefore, adenosine receptor occupancy results in PI3-K activation and Akt phosphorylation, although the intermediate steps are not yet known.

It is unclear why only the A_{2b} receptors appear to protect the postconditioned heart. The untreated heart produces and releases adenosine during the prolonged coronary occlusion, and, therefore, one might wonder why these hearts are not protected. The A_{2b} receptor is a very low-affinity receptor and the adenosine concentration even during ischemia may not be sufficient to populate this receptor to the degree required for protection. NECA, on the other hand, is a more potent agonist of the critical A_{2b} receptor. Kin et al. [7] measured adenosine in the effluent of their postconditioned hearts and found it to be decreased. They proposed that the postconditioned heart might somehow be retaining adenosine in the tissues, but the mechanism for this is unprecedented. Perhaps postconditioning somehow activates pathways that increase the affinity of the receptors so that they can be occupied by the heart's endogenous adenosine. Indeed, PKC activation has been reported to increase A_{2b} responses in some cell systems [27,28] and we now find that PKC activation is required for postconditioning to elicit protection. Although PKC may activate a myriad of kinases, an intriguing possibility is that it phosphorylates the A_{2b} receptor to increase its sensitivity.

Inagaki et al. [29,30] have reported that specific inhibition of PKC-δ at reperfusion decreases infarct size. Our present observation that PKC activation at reperfusion is protective is obviously not consistent with that paradigm. Perhaps activation of all isoforms by PMA activates a previously unsuspected protective isoform, while inhibition of all isoforms with chelerythrine blocks some additional protective isoform resulting in the deleterious effect that we observed.

In conclusion, our study demonstrates for the first time that at least some protective strategies introduced shortly before reperfusion and ischemic postconditioning are acting through the adenosine A_{2b} receptor. Furthermore, binding of the A_{2b} receptor at reperfusion results in activation of at least one prosurvival kinase, PI3-K. Finally, PKC must also be activated during postconditioning to elicit protection, perhaps by increasing the sensitivity of the A_{2b} adenosine receptor. Hence, PKC activation is upstream of A_{2b} receptor occupancy.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2006.02.014.

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